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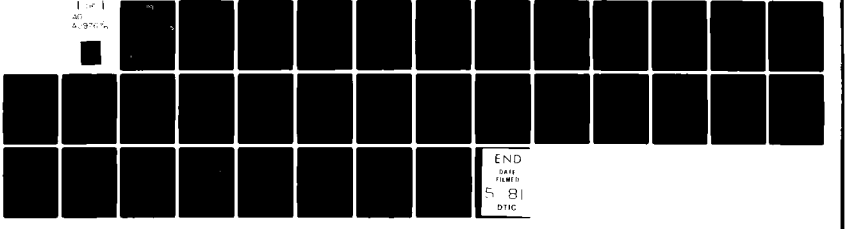

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The Physiological Bases for Microbial Barotolerance

by

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31 March 1981

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REPORT FOR PERIOD FROM 1 APRIL 1980 TO 31 MARCH 1981

I. Introduction

The long-range goals of our barophysiology program are to define the bases for differences in barotolerance among microorganisms in terms of the pressure sensitivities of specific biochemical or physiological processes and to explore ways to use pressure advantageously in basic and applied microbiology. These goals have been expanded somewhat to include acquisition of knowledge of the mechanisms by which high-pressure gases affect cell growth.

The report will be divided into two main sections describing progress in the study of the effects of compressed gases and other narcotic agents and progress in the program on the biological effects of hydrostatic pressure.

II. Compressed Gas Effects

A. Experiments with tissue-culture cells. Last year, we reported the results of preliminary experiments on the inhibition of the growth of 3T3 mouse fibroblasts in monolayer tissue culture. These cells are extremely sensitive to gases such as N_2O, and the dose for 50% inhibition of growth $\{ID_{50}\}$ was found to be less than one atmosphere {atm}. This extreme sensitivity limits the usefulness of these cells for further experimentation, and so we tested other cell lines for sensitivity to N_2O.

We have now completed initial work with two other cell types in tissue culture - HeLa cells, derived decades ago from a human tumor, and RRP 104c10 cells, which are haploid cells from the frog Rana pipiens. Both cell types are hearty and amenable to the manipulations needed for hyperbaric experimentation. Moreover, there is some preliminary work reported previously by Bruemmer et al. {1967} on the responses of HeLa cells to compressed gases. The frog cells offer the advantages of an optimal growth temperature close to room temperature and the haploid state, which allows for direct isolation of hypersensitive or resistant mutants.

Fortunately, these cells are more gas resistant than are 3T36D mouse fibroblasts. Fig. 1 shows dose-response curves for HeLa cells exposed to helium, argon and N_2O. Here the response is the yield after 6 days of growth, and the dose is in atm. From this data, we can estimate 50% inhibitory doses for growth of about 90 atm for He, 60 atm for Ar and 3 atm for N_2O. Fig. 2 shows typical growth curves obtained in a single experiment. It can be seen that the gases, here N_2O, act to slow growth and to reduce the culture yield. Therefore, superficially, the responses of HeLa cells in culture are similar to those of the bacterium Streptococcus faecalis.

Fig. 1. Inhibition of the growth of HeLa cells in monolayer tissue culture by N_2O, Ar and He. Cells were grown in Leighton tubes at 37°C in Dulbecco's MEM medium with an initial pH of 7.4 and 2 mM glutamine, 25 mM HEPES buffer and 10% calf serum. Tubes were inoculated with approximately 5×10^4 cells/ml. The cells were allowed to attach to the plastic surface of the Leighton tubes, the tubes were placed in standard pressure chambers and compressed with gases from commercial tanks. The caps of the tubes were not tightened so that ready exchange of gases was possible. The air initially present in the cylinders was not flushed out. The HEPES buffer spares the need for bicarbonate buffering. Control, 1-atm cultures were handled in exactly the same manner except that compressed gases were not used. All chambers were placed on their sides so that the monolayers were always covered by growth medium. At intervals, the chambers were slowly decompressed and opened so that tubes could be removed. Some cultures were compressed and decompressed a number of times, but this procedure did not appear to affect adversely cell growth or to result in intracellular bubble formation. The cells were removed from the monolayer by means of trypsinization. Then, total numbers of cells per ml of medium were determined by use of a standard hemocytometer.

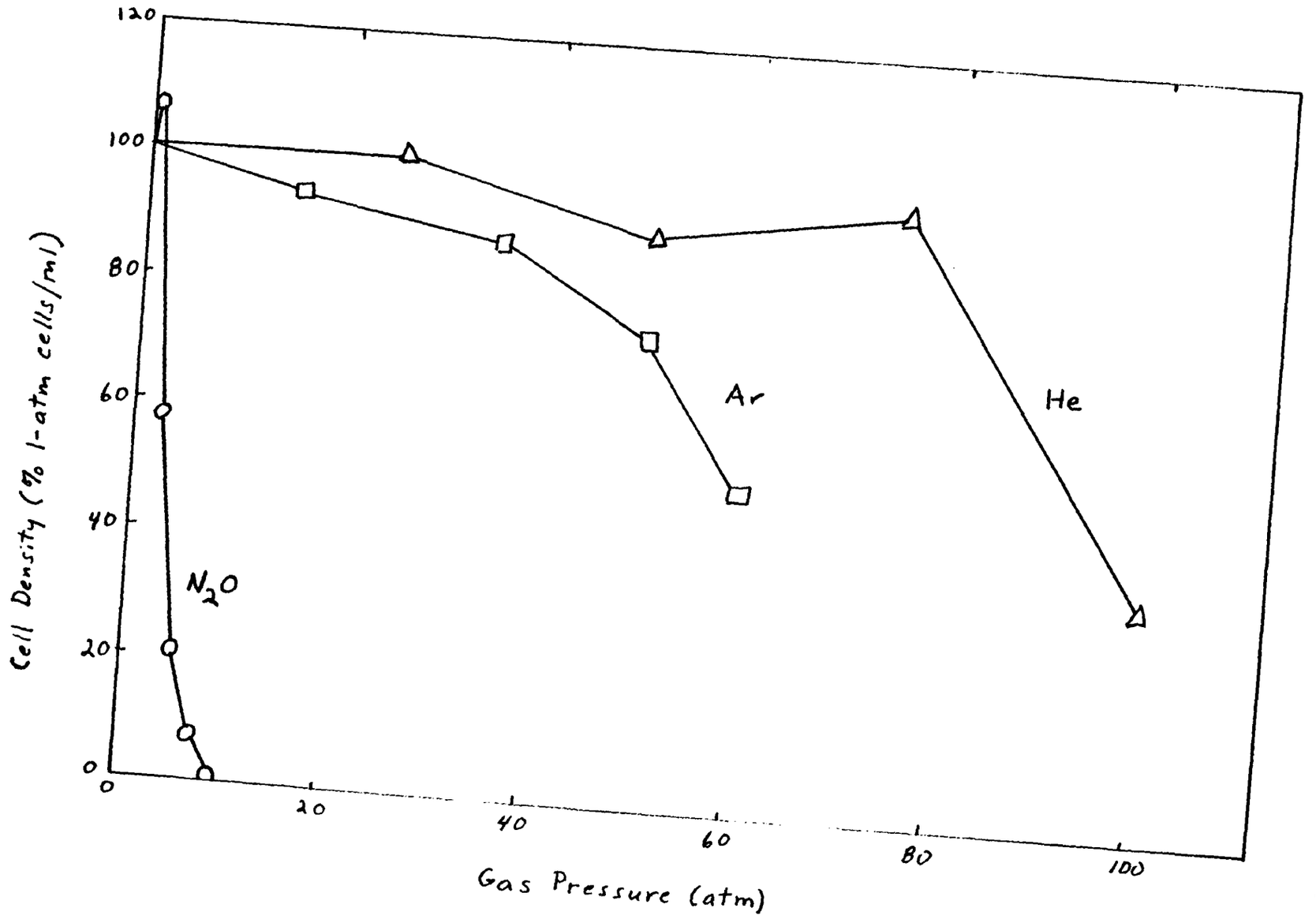
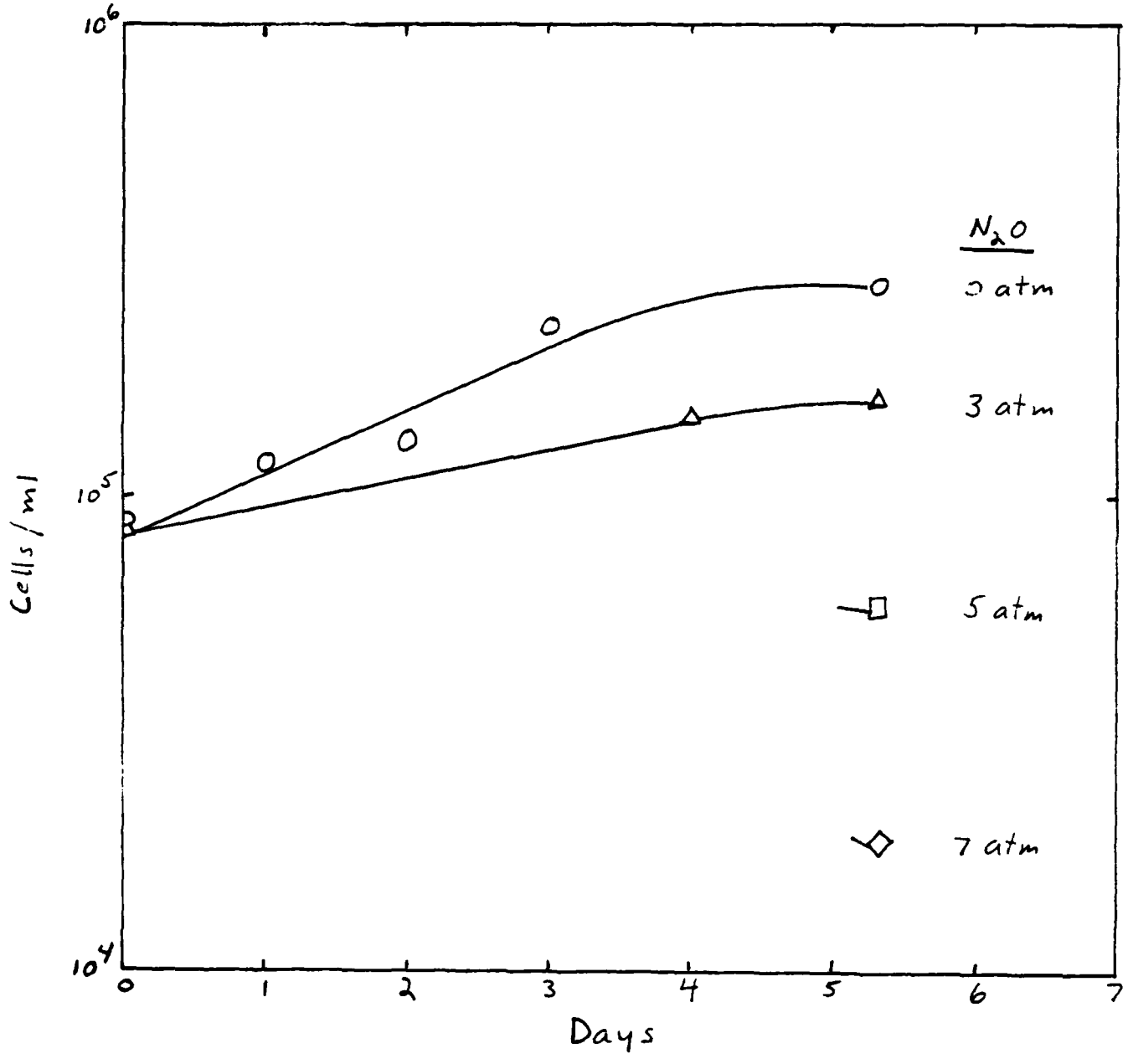

120
100
80
60
40
20
0
0
20
40
60
80
100
Cell Density (% 1-atm cells/ml)
Gas Pressure (atm)
N_2O
Ar
He

Fig. 2. Inhibition of growth and killing of HeLa cells by N_2O. See Fig. 1 legend for experimental details.


10^6
10^5
10^4
Cells / ml
0
1
2
3
4
5
6
7
Days
N_2O
0 atm
3 atm
5 atm
7 atm

It is apparent also from the data presented in the figures that the gases can be lethal to HeLa cells. The final counts shown in Fig. 2 for cultures exposed to 5 and 7 atm N_2O are lower than initial counts. The cells not only were killed but must also have undergone autolysis. Exposure to 9 atm N_2O resulted in a final count of only about 5×10^3 cells/ml, or over 90% reduction from the initial count. A similar lytic response has been described in past project reports for bacterial cells. Because of our earlier experience with 3T3 fibroblasts, we purposely used high inocula of HeLa cells in these experiments. Presumably, the gases would be even more effective if smaller inocula were used. However, we have just started experiments to define the relationship between inoculum size and sensitivity to compressed gases, and data will not be available until the next report.

We have initially used a somewhat different approach to investigate the responses of frog cells to the gases. With the frog cells, we have looked at colony formation after long-term incubation at 25°C. The data obtained then is in terms of colony forming units as a function of gas pressure. With this approach, it has been possible to define 50% inhibitory doses of >100 atm for He, 52 atm for N_2, 32 atm for Ar and 1 atm for N_2O. Therefore, these cells appear to have sufficient resistance to the gases so that it will be possible to carry out a series of experiments with gas combinations to determine whether or not the additive action rule applies to growth inhibition of frog cells as it applies to narcotic responses.

The work planned for the upcoming support period includes completion of the experiments described above to establish firmly the potency series for inhibition of HeLa and RRP 104c10 cells, and of special significance, experiments with mixtures of gases to find out if the additivity rule for narcosis is violated for growth effects with animal cells as it is with microbial cells.

The data obtained to date with animal cells suggest that their responses to compressed gases are somewhat didifferent from the responses of microbial cells. For example, N_2 or Ar alone are not inhibitory for microbial growth. In fact, these gases mute the effects of hydrostatic pressure, even though they can potentiate the actions of agents such as N_2O. However, with the tissue-culture cells, N_2 and Ar do appear to be growth inhibitors. We are now anxious to test the effects of combined gases to find out if there are other differences in the responses of microbial and animal cells. If there are difference, then we shall have to start thinking in terms of unique targets in animal cells.

B. Experiments with microbial cells.

1. Growth inhibition by liquid anesthetics. The net conclusion of a great deal of work on growth inhibition by liquid anesthetics is that these agents fall fall into at least three separate, definable classes when considered together with gaseous anesthetics such as N_2O. In fact, it seems that one cannot reasonably prepare predictive plots relating potency for growth inhibition to simple physico-chemical parameters such as lipid solubility, as one can do for anesthesia and other narcotic responses. This conclusion is in some ways disappointing because it indicates a very complicated picture for growth modification by narcotic agents. However, from a therapeutic perspective, the conclusion is a positive one because it indicates great versatility for manipulating host responses to the various agents and combinations of the agents.

During the support period, we have determined that ketamine, a cationic anesthetic agent, can inhibit growth of microorganisms. This agent behaves in general like halothane or methoxyflurane in its inhibitory action and has approximately the same potency. Ketamine at a 6 mM concentration can completely inhibit growth of E. coli B in complex medium. A concentration of about 10 mM was required for complete stoppage of growth of S. cerevisiae. Moreover, the inhibitory action of ketamine was increased by hydrostatic pressure, as is the action of halothane or methoxyflurane. {See Table 1 for data on the growth inhibitory action of these anesthetics for S. cerevisiae.} Therefore, it appears that the actions of these agents cannot be predicted by use of the critical volume hypothesis, and one must consider more complex hypotheses such as the multi-site proposal of Wardley-Smith and Halsey {1979}. Helium at a pressure of 20 atm was found to enhance the inhibitory actions of ketamine and halothane, as it potentiates the inhibitory action of N_2O.

We have also continued work on the inhibitory actions of aliphatic alcohols, especially heptyl alcohol, for microbial growth. The alcohols are good examples of a second class of agents. As indicated in our last report, low hydrostatic pressures of 100 or 200 atm act to reverse the inhibitory effects of heptyl alcohol. However, higher pressures act to enhance the inhibitory effects. Helium, nitrogen and argon had essentially no effect on the inhibitory action of heptyl alcohol.

In all, then, there seem to be three definable classes of agents. N_2O is an example of the first class. Its action is reversed by hydrostatic pressure but potentiated by helium, argon or nitrogen. The aliphatic alcohols are examples of a second class. Low pressures reverse their inhibitory actions, higher pressures potentiate, and He, Ar or N_2 have no effect. Finally, a third class of agents is

exemplified by ketamine, halothane and methoxyflurane. Their actions are enhanced by hydrostatic pressure and also by helium pressure, as indicated by the data in Table 1. A more detailed view of the interactions of halothane and hydrostatic pressure in inhibiting growth of E. coli B is presented in Fig. 3.

2. Effects of temperature on growth inhibitory potential of nitrous oxide for E. coli. In the report of last year, we presented data to show that growth inhibition of S. cerevisiae by N_2O is affected in a complex way by changes in growth temperature with an optimal temperature for resistance of about 24°C and decreased resistance at higher or lower temperatures. This pattern can be contrasted with that shown by narcotic effects, which generally show monotonic changes with increasing sensitivity in response to decreasing temperature.

Fig. 4 presents data on changes in ID_{50} of N_2O for growth inhibition of the bacterium E. coli over the temperature range from 12 to 42°C. It is again apparent that there is a temperature, or range of temperatures, for maximal resistance to N_2O and that resistance is decreased at higher and lower temperatures. Growth yield of E. coli was found not to vary from 12 to 42°C. However, the rate of growth was affected by temperature at the extremes of this range. The data presented here are for yield or A_{700}^{max}. The changes in resistance found for the bacterium are not as marked as those found for the yeast.

The protozoan Tetrahymena thermophila was found to behave much as did E. coli and S. cerevisiae. For example, average percentage inhibitions of growth caused by 2 atm N_2O at 18, 24, 30, 37 and 40°C were, respectively, 59, 30, 27, 68 and 75. If one considers the increasing values for the Ostwald coefficient as temperature decreases, the organism was most resistant at about 24°C. Unfortunately, growth of this organism, with an optimum at about 30°C, was more temperature sensitive than was that of the yeast or E. coli. Therefore, there are complexities in direct interpretation of the data.

We also determined the effects of temperature on the sensitivities of E. coli and S. cerevisiae to a liquid anesthetic, n-heptanol. Temperature effects were small with this agent. For E. coli, ID_{50} values at 18, 24, 30 and 37°C were, respectively, 0.046, 0.057, 0.034 and 0.035% {v/v}. For S. cerevisiae, ID_{50} values at 18, 24, 30 and 37°C were, respectively, 0.025, 0.034, 0.034 and 0.029% {v/v}. Again, for each organism, there appears to be an optimal temperature for resistance, but as mentioned, the variations in sensitivity with change in temperature were not as great for heptanol as for N_2O.

Table 1 - Effects of hydrostatic pressure and helium pressure on the growth inhibitory actions of ketamine, methoxyflurane and halothane for *Saccharomyces cerevisiae*[a]

Agent	Hydrostatic pressure (atm)	Helium pressure (atm)	A_{700}^{max} (% 1-atm control)
None	1	0	100
6 mM methoxyflurane	1	0	74
6 mM methoxyflurane	100	0	23
6 mM methoxyflurane	1	20	102
None	1	20	105
None	100	0	81
2 mM ketamine	1	0	83
2 mM ketamine	100	0	47
2 mM ketamine	1	20	70
None	1	20	103
None	100	0	73
1 mM halothane	1	0	98
1 mM halothane	100	0	39
1 mM halothane	1	20	56
None	1	20	100
None	100	0	70

[a]Cultures were inoculated with a 5% inoculum of an overnight culture in tryptone-glucose-Marmite medium plus 162 µg ampicillin per ml and incubated at 24°C. Absorbancy, which is directly related to biomass per unit volume of culture, was assessed with a Beckman DU spectrophotometer set for light of 700 nm wavelength. The values indicated are maximal absorbancy values relative to the 1-atm control value. Techniques for applying hydrostatic pressure and gas pressure have been described in detail previously.

Fig. 3. Inhibition of growth of E. coli B in tryptic-soy broth plus 0.1% {w/v} KNO_3 at 24°C by 1.57 and 3.14 mM halothane at 1, 50 and 150 atm hydrostatic pressure.

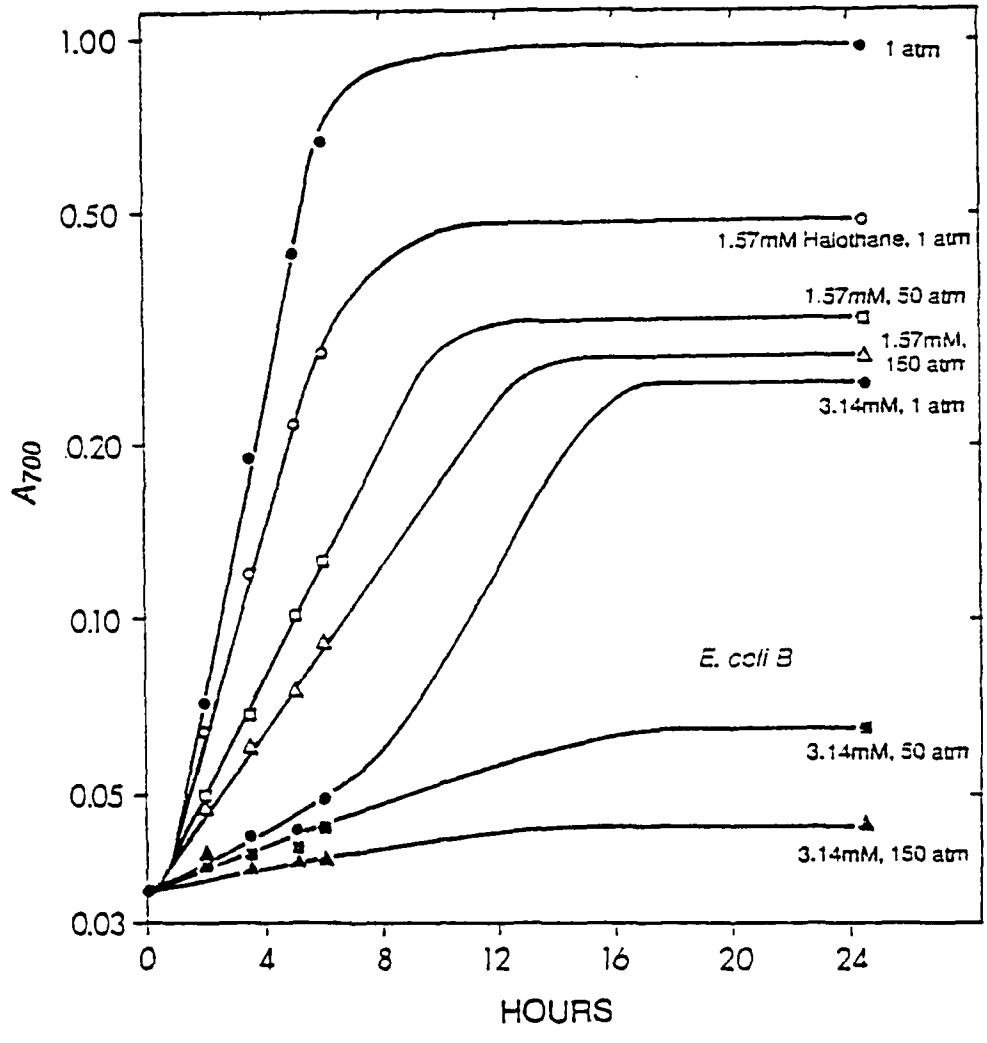
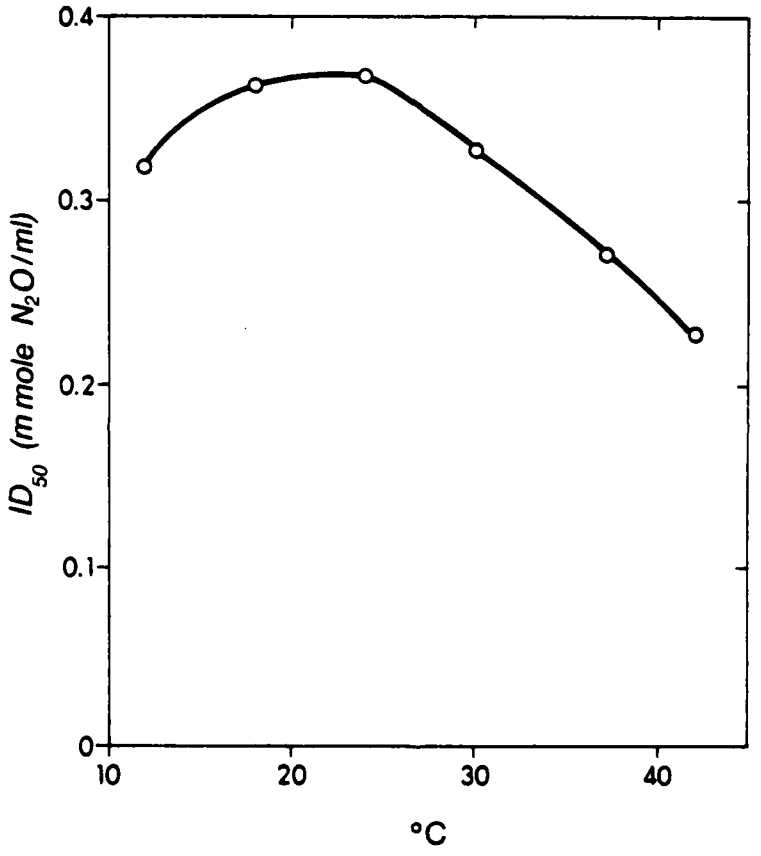

1.00
0.50
0.20
0.10
0.05
0.03
A_{700}
0
4
8
12
16
20
24
HOURS
1 atm
1.57mM Halothane, 1 atm
1.57mM, 50 atm
1.57mM, 150 atm
3.14mM, 1 atm
3.14mM, 50 atm
3.14mM, 150 atm
E. coli B

Fig. 4. Effect of temperature on the sensitivity of E. coli B to the growth inhibitory action of N_2O. Cultures were grown in tryptic-soy broth (Difco. Co., Detroit, Michigan) plus 1% (w/v) KNO_3.


0.4
0.3
0.2
0.1
0
10
20
30
40
°C
ID_{50} (m mole N_2O/ml)

3. Effects of compressed gases on endospore formation and germination - examples of prokaryotic differentiation. Hydrostatic pressure is a potent inducer of the process of germination in bacterial endospores. {See the next section of hydrostatic pressure effects.}. However, compressed gases have just the opposite effect - they inhibit germination. Moreover, data obtained during the past year indicate that they can also inhibit the process of spore formation. These two processes, spore formation and germination, are widely studied examples of prokaryotic differentiation. Studies of the effects of compressed gases and of hydrostatic pressure on differentiation are logical extensions of our previous growth studies.

Table 2 presents data on the effects of compressed gases on germination of spores of Bacillus megaterium ATCC 19213 induced with the chemical germinants 1 mM L-alanine and 1 mM inosine. Helium had little effect, even at 100 atm, although it may have slowed germination somewhat. We did not test the effects of higher pressures of helium because these higher pressures would be germinating pressures and would make interpretation of the data difficult. However, N_2, Xe and N_2O all could be used to completely suppress germination. The suppression proved not to be irreversible. Decompression and subsequent exposure of the spores to chemical germinants or to 748 atm hydrostatic pressure resulted in normal germination. Halothane, methoxyflurane and aliphatic alcohols also were inhibitory for chemically induced germination. For example, complete suppression of germination was obtained with 20 mM halothane or methoxyflurane. These concentrations for inhibition of germination are only slightly higher than the concentrations needed for complete inhibition of growth.

As mentioned, germination can be induced by hydrostatic pressure without any need for chemical germinants. The gases listed in Table 2 had little or no effect on germination induced by 748 atm hydrostatic pressure. Previously, Enfors and Molin {1978} reported on the inhibition of germination of Bacillus cereus spores by inert gases, O_2 and CO_2. They also reported that pressure could reverse the inhibitory effects of the gases. Therefore, it seems that our findings regarding pressure germination can be interpreted in two ways. It may be that germination induced by chemicals is different from that induced by pressure, presumably in the early steps involving membrane triggering. Alternatively, there could be no difference but germinating pressures could also be those which can inhibit gas effects. We current favor the former notion. However, additional experimentation is needed

Table 2 - Effects of compressed gases on chemically induced germination of endospores of *Bacillus megaterium*[a]

Compressed gas	% germination	
	1 hour	24 hours
None	55	74
100 atm He	39	73
4 atm Xe	2	4
2 atm N_2O	0	1

[a] *B. megaterium* ATCC 19213 spores were harvested by centrifugation from the sporulation medium of Slepecky and Foster (1959) and washed free of cell debris. For germination, they were resuspended in 67 mM potassium phosphate buffer. L-alanine and inosine were then added to give final concentrations of 1 mM. The suspensions were immediately placed in pressure chambers, compressed and incubated at room temperature. Subsequently, samples were taken and observed by means of phase microscopy. Spores which appeared phase dark were considered to have germinated.

to distinguish between the two alternatives.

The gases were found also to inhibit the process of spore formation. For these experiments, B. megaterium ATCC 19213 cultures were incubated aerobically at 30°C until the early stationary phase. They were then washed once with cold water and resuspended in 1 mM $CaCl_2$ solution to allow them to undergo so-called endotrophic sporulation at room temperature. Helium at a pressure of 100 atm actually enhanced the endotrophic sporulation process. However, 2 atm N_2O, 20 atm O_2 or 30 atm N_2 completely suppressed sporulation. Ar at a pressure of 5 atm very much reduced sporulation, and after 48 hours of incubation, the population showed only about 20% sporulation, compared with 85% sporulation in the control 1-atm suspension. It appears then that compressed gases can inhibit not only growth of prokaryotes but also differentiation. However, the actions on differentiation appear to be significantly different than those affecting growth. Ar and N_2 are not effective growth inhibitors but they can inhibit sporulation and germination.

III. Hydrostatic Pressure Effects

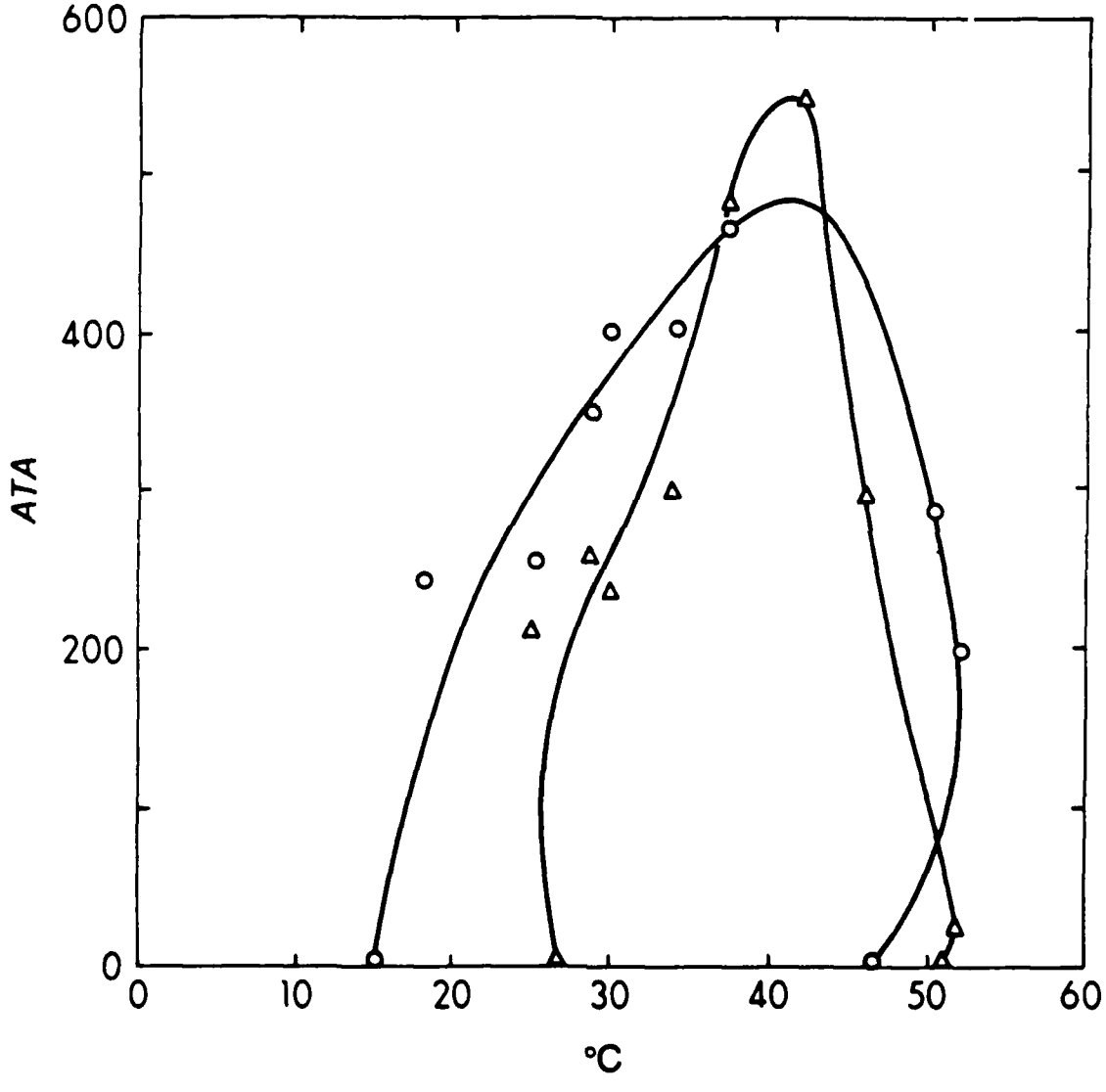
A. Parametric analyses of pressure interactions affecting growth. In previous reports, we have discussed the importance for marine ecology of the interactions of various environmental parameters with hydrostatic pressure. Moreover, we have examined the process of pressure denaturation of proteins as a model for designing growth studies. The data of Zipp and Kauzmann (1973) for pressure denaturation of metmyoglobin as a function of pH and temperature has been particularly useful. They considered denaturation to be a highly cooperative reaction with only two molecular forms involved - the native form and the denatured form. Therefore, when the concentrations of the two forms were equal, ΔG for the reaction would be equal to zero. They then determined various conditions of pH, temperature and pressure at which ΔG was equal to zero. Finally, they prepared plots of the data for $\Delta G = 0$. For example, a plot in the temperature-pH plane of the values of temperature and pH at which $\Delta G = 0$ for some particular pressure would outline a contour. Within this contour would be conditions of pH and temperature under which the native state was favored. Outside of the contour would be conditions under which the denatured state was favored. An important point here, is that they obtained closed contours from these sorts of plots. Obviously, one would also obtained closed contours for plots in the pressure-temperature or pressure-pH plane. The closed contours indicate clearly that for any particular protein, there is a high pressure denaturation process, but there is also a low pressure denaturation process. In other words, the native state is favored only within certain limits of pressure.

We have followed this same approach in studying the interactions of pressure with other environmental factors affecting growth. We had first to define some standard, reference state. For the data presented in Fig. 5, the standard was the extent of growth at 37°C and one atm. It would also have been possible to have chosen some rate of growth as a standard, and in fact, we have prepared such plots relating growth rates under various conditions to the standard. However, here it is more convenient to present data for extent of growth. For the two test bacteria, S. faecalis and Bacillus licheniformis, rate and extent are related directly. For each point plotted in Fig. 5, we have first to prepare plots of extent of growth versus pressure at a variety of temperatures. Then, from the data for any one temperature, we could determine the pressure at which the extent of growth was equal to that at one atm and 37°C. This value would then be plotted in the pressure–temperature plane of Fig. 5. The contours in Fig. 5 are not complete because we had no means in the laboratory to examine growth at negative pressures. Therefore, the contours stop at the zero pressure line. However, it does appear that closed contours would have been obtained if we could have performed the negative-pressure experiments. Presumably, if we had been working with an obligately barophilic bacterium, the entire contour would be in the positive pressure region of such a plot. In fact, this sort of plot is really required to define an organism as an obligately barophilic organism.

One of the major premises we have been developing over the past few years is that many environmental parameters affect the barosensitivity of an organism. Generally, in natural environments, conditions are not optimal for growth, and it seems that in these circumstances, even low pressures may have major ecological effects. Clearly, there is need to question the view that hydrostatic pressure is a major ecological influence only in the very deep ocean where pressures of many hundreds of atmospheres occur. It seems that in the shallower parts of the ocean, in the regions of the continental shelves and slopes, pressure could play a major role in determining the distribution and activities of microorganisms.

The work currently underway on this aspect of the project is aimed at defining the relationships between hydrostatic pressure and ionic strength affecting the growth of the test organisms. We had previously described the striking interactions between pressure and pH affecting growth of many organisms. Pressures of some hundreds of atmospheres markedly reduce the pH range for growth. Moreover, extremes of pH markedly reduce the barotolerance of microorganisms. The view we are now developing is that these interactions involve membrane ATPases.

Fig. 5. Interaction of pressure and temperature affecting bacterial growth. Data shown are for S. faecalis {O} growing in tryptone-glucose-Marmite broth and for B. licheniformis {Δ} growing in the same broth supplemented with 0.1% KNO_3. Each point indicates pressure-temperature conditions under which the extent of growth is equal to that at one atm and 37°C.

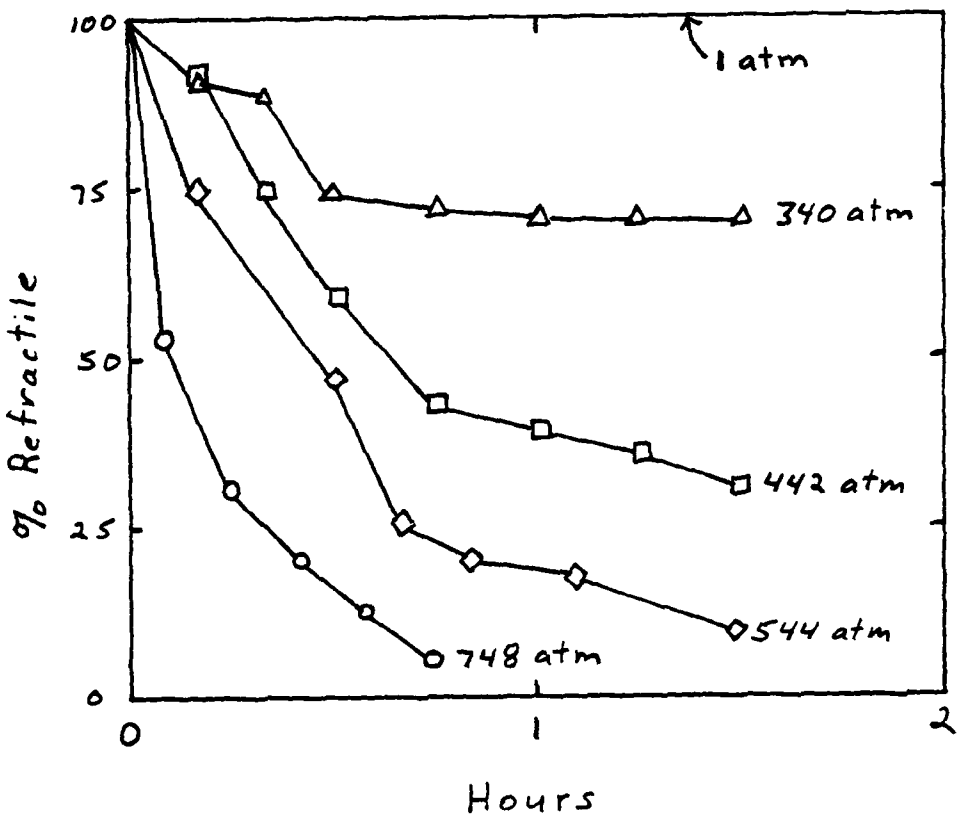

600
400
200
0
ATA
0
10
20
30
40
50
60
°C

B. Pressure-induced germination of bacterial endospores. It has been known for many years that hydrostatic pressure can trigger germination of bacterial endospores. {See Gould and Sale, 1972, for a review of the initial work.} This induction of germination by a physical agent is of interest in relation to the study of the basic biochemical/biophysical mechanisms for breaking of dormancy. It is also of practical interest because the inactivation of bacterial endospores is a major industrial activity, especially in the food or medical products industries. We have an interest in the process also in relation to marine ecology. Microbiological examination of samples obtained from deep marine sediments has indicated that sporeformers are major components of sediment flora, even though they are rare in the supratending water column. It seems that some of the sporogenous bacteria of the Bacillus and Clostridium genera must be living and growing in the deep sediments. However, others must be there only in the dormant state. For example, Bacillus stearothermophilus bacteria have been isolated repeatedly from deep marine sediments. These organisms are obligate thermophiles and could not be growing in the cold environment of the deep ocean. Therefore, they must be there in the dormant spore form, which would have settled to the bottom from the surface. One wonders how these spores manage to remain dormant in high-pressure environments, especially since B. stearothermophilus spores are noted for being readily triggered to germinate by hydrostatic pressure.

For our initial work on pressure induced germination, we chose Bacillus megaterium ATCC 19213 because of the ease with which its mineral content can be manipulated. Moreover, it is an easy matter to obtain large crops of clean spores of this organism by growing it in the sporulation medium of Slepecky and Foster {1959}. We plan to do some work at a later date with spores of B. stearothermophilus, but it is more difficult to obtain the large crops of spores we need with this organism.

Fig. 6 shows data obtained in experiments designed to determine the inducing action of hydrostatic pressure for germination of isolated spores of B. megaterium ATCC19213. For these experiments spores were suspended in 67 mM potassium phosphate buffer, pH 6.8. They were then compressed, and at intervals, samples were taken and observed with the phase microscope and a Petroff-Hausser counting chamber. Phase dark spores were considered to have germinated. The experimental temperature was approximately 24°C. It is apparent that hydrostatic pressure does induce germination. A pressure of 340 atm induces about 25% germination in the population, and a pressure 748 atm induces almost complete germination.

Fig. 6. Germination of B. megaterium ATCC 19213 spores in 67 mM potassium phosphate buffer pH 6.8 at 24°C caused by hydrostatic pressure. Spores remaining refractile in phase microscopic appearance were considered not to have germinated.


100
75
50
25
0
% Refractile
0
1
2
Hours
1 atm
340 atm
442 atm
544 atm
748 atm

Our initial thought was that the low temperature of the ocean might act to inhibit pressure-induced germination. We found that germination of B. megaterium spores was, in fact, very much slowed by ice-bath temperature, even at 748 atm. The response which was complete in 2 hours at 24°C occurred only over a period of days at close to 0°C. However, germination did occur at low temperature, and the time for germination was short compared with the very long periods of time that would be required for sedimentation of micron sized particles in the ocean.

There are still two possibilities to be considered regarding this subject. Possibly, because B. stearothermophilus is a thermophile, temperatures of about 0°C would have a greater retarding effect than they do for B. megaterium. We doubt that this view is correct because of various reports that centrifugation of B. stearothermophilus spores in a refrigerated centrifuge can induce germination. Presumably, this germination is caused by the hydrostatic pressure in the centrifuge tubes.

Another possibility has to do with heterogeneity in spore populations. Whenever a spore population is induced to undergo germination, chemically or with hydrostatic pressure, there is always a small fraction which remains refractile. Possibly, it is these cells which survive in the deep. They seem to be refractory to simple germinants but can germinate in complex growth media. What factors determine this extreme state of dormancy in some members of the population? Presumably, the factors are mainly phenotypic rather than genetic since germination and growth of these extreme forms in complex media result in a population that is as heterogeneous as populations obtained from ordinary spores.

During the past few years, we have developed a technique for completely exchanging the ions of B. megaterium spores without killing the cells. Briefly, the procedure involved use of aqueous suspensions containing 2 g, wet weight, of spores per 50 ml. These were titrated to a pH value of 2, over 2 to 3 hours, with intermittent addition of 0.02 N HCl solution and then incubated at 60°C for up to 18 hours to obtain protonated or H spores. H spores were found to be nearly devoid of calcium or other minerals but viable. The spores could be remineralized by back-titration with appropriate bases and incubation of the suspensions at 60°C for up to 72 hours. All of the desired salt forms could be prepared. The extents of exchange were such that core electrolytes as well as those in enveloping structures must have been involved.

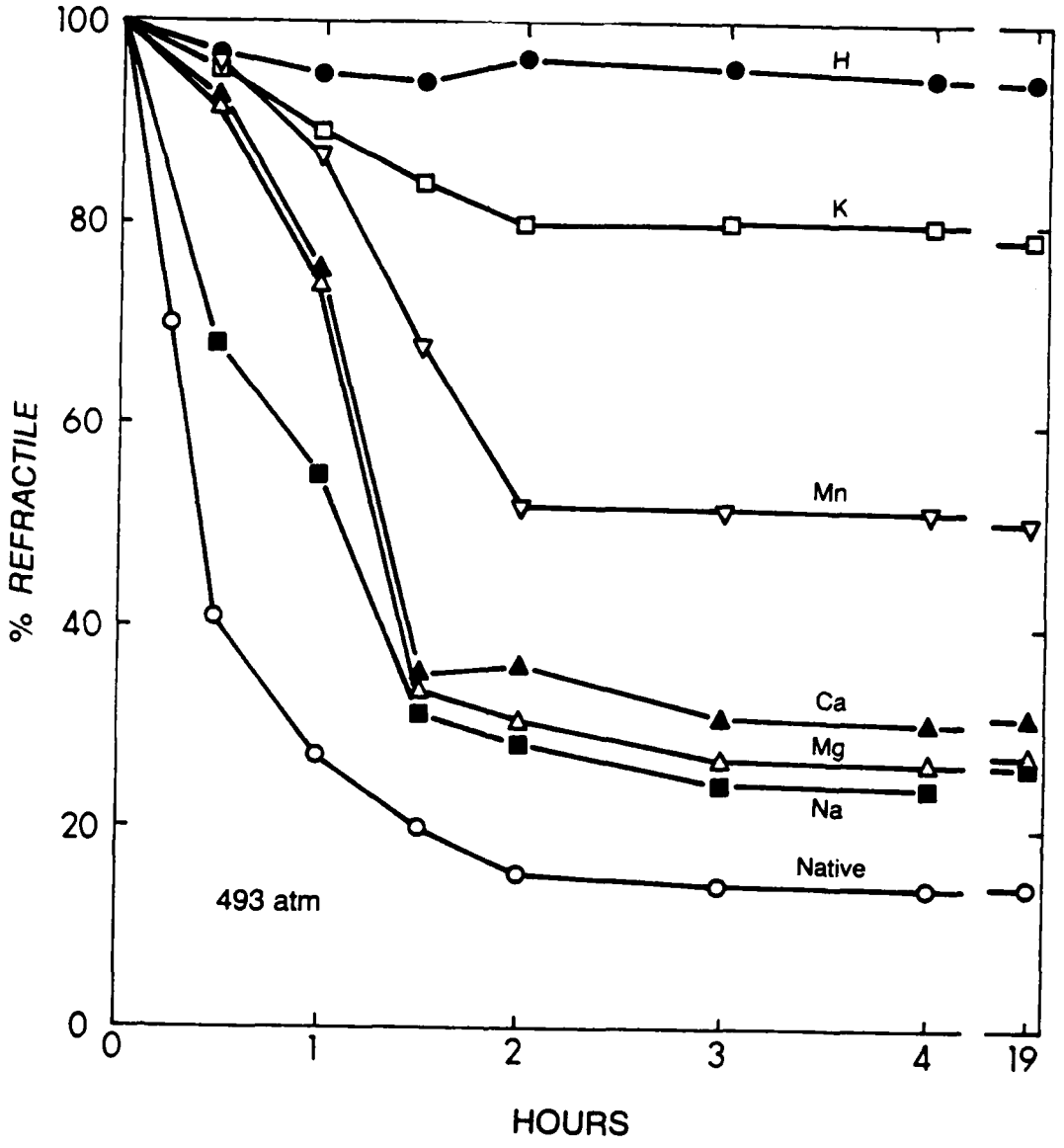
Experimental results obtained to date indicate that the mineral contents of the spores have major effects on responses to the germinating action of pressure. The data presented in Fig. 7 show that native spores suspended in phosphate buffer at pH 6.8 germinate rapidly after exposure to 493 atm hydrostatic pressure. In contrast, the hydrogen form of the spores is refractory to pressure germination. This same form is the most sensitive to heat of the various salt forms. In other words, the hydrogen form is the most heat sensitive but the most pressure resistant. The other salt forms proved to be intermediate in pressure sensitivity. The Na, Mg and Ca forms were all only slightly more resistant to pressure than was the native form. The Mn form was considerably more resistant, and the K form was nearly as resistant as the H form.

Initially, we thought that the hydrogen form may be superdormant. However, hydrogen spores do respond to chemical germinants and so they are clearly not superdormant.

C. Other projects. During the support period progress was made also in the isolation of membrane vesicles that will be used for studies of the effects of hydrostatic pressure on the proton transporting functions of the membrane ATPase of S. faecalis. Hopefully, by the final report we shall be able to present the results of experiments carried out with these vesicles.

Progress has been made also on the study of ultrasonic absorption by bacterial cells. Our major finding to date is that bacterial endospores do not have abnormally high absorption coefficients as we thought they would. The final report will present data on this subproject, and hopefully, an interpretation of the data in biophysical terms.

Fig. 6. Pressure sensitivities of various salt forms of endospores of B. megaterium at 24°C.


100
80
60
40
20
0
% REFRACTILE
0
1
2
3
4
19
HOURS
H
K
Mn
Ca
Mg
Na
Native
493 atm

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Our work in the past year has been directed to two related topics. One has to do with the effects of hydrostatic pressure on microbial growth and metabolism. The other is concerned with the effects of compressed gases and other narcotic agents on growth and differentiation of microorganisms and on growth of tissue-culture cells.

One of the major components of the hydrostatic pressure project was a parametric analysis of the interactions of pressure with other environmental factors affecting microbial growth. For this analysis, we chose a set of

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standard conditions, specifically 37°C, 1 atmosphere pressure and tryptone-glucose-Marmite medium, determined rate and extent of growth under these conditions, and then determined the various other sets of parametric values at which these standard growth responses occur. We were then able to prepare plots, for example, in the pressure-temperature plane of these sets. Such a plot outlines a contour, which gives a visual impression of the interactions between pressure and temperature affecting growth. During the year, we carried out such analyses for growth of Streptococcus faecalis and Bacillus licheniformis.

The other major project in microbial barobiology was on the effects of pressure on prokaryotic differentiation, specifically on pressure induced germination of endospores of Bacillus megaterium ATCC 19213. We were able to prepare various salt forms of the spores by means of an acidification-neutralization procedure that results in essentially complete exchange of minerals but, amazingly, little or no loss in viability in the population. We found that the hydrogen form is extremely resistant to pressure induced germination. The K form was less resistant, followed in series by the Mn, Ca, Mg, Na and native forms. A pressure of 493 atm was sufficient to induce over 80% germination in a population of native spores in 2 hours. In contrast, this same pressure induced no germination in a population of fully viable hydrogen spores even after 19 hours of incubation.

Much of the effort in our work on the biological effects of compressed gases was focused on animal cells in tissue culture, specifically HeLa cells and RRP 104c10 haploid, frog cells. The gases were inhibitory for growth of both cell types, and we were able to determine 50% inhibitory pressures of 90 atm for He, 60 atm for Ar and 3 atm of nitrous oxide for the HeLa cells. Our major task now is to find out if these animal cells respond to the gases in the same manner that microbial cells do. In past research, we have found that modification of microbial growth by compressed gases cannot be viewed as a narcotic response but is in a different class of responses.

This year we continued our study of the variation with temperature change in the growth inhibitory potency of nitrous oxide, now with E. coli and Tetrahymena. As we reported last year for yeast, there appears to be for each organism a temperature for maximal resistance to the gas with greater sensitivity at either higher or lower temperatures. This pattern is different from that usually found for simple narcotic responses.

Also, we have completed work on potency determinations for a wide variety of narcotic agents as they affect microbial growth. The net conclusion is that the agents fall into a number of definable classes and that one cannot reasonably relate growth inhibition potency simply to some physicochemical parameter such as lipid solubility.

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